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Bovine Viral Diarrhea Virus: Prevention of Persistent Fetal Infection by a Combination of Two Mutations Affecting E^{rns} RNase and N^{pro} Protease▽

Gregor Meyers,[1]* Andreas Ege,[1] Christiane Fetzer,[1]† Martina von Freyburg,[1]‡ Knut Elbers,[2] Veronica Carr,[3] Helen Prentice,[3] Bryan Charleston,[3] and Eva-Maria Schürmann[1]

Institut für Immunologie, Friedrich-Loeffler-Institut, D-72001 Tübingen, Germany[1]; Boehringer Ingelheim Vetmedica GmbH, D-55216 Ingelheim am Rhein, Germany[2]; and Institute for Animal Health, Compton, Newbury, Berkshire RG20 7NN, United Kingdom[3]

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Different genetically engineered mutants of bovine viral diarrhea virus (BVDV) were analyzed for the ability to establish infection in the fetuses of pregnant heifers. The virus mutants exhibited either a deletion of the overwhelming part of the genomic region coding for the N-terminal protease N^{pro}, a deletion of codon 349, which abrogates the RNase activity of the structural glycoprotein E^{rns}, or a combination of both mutations. Two months after infection of pregnant cattle with wild-type virus or either of the single mutants, the majority of the fetuses contained virus or were aborted or found dead in the uterus. In contrast, the double mutant was not recovered from fetal tissues after a similar challenge, and no dead fetuses were found. This result was verified with a nonrelated BVDV containing similar mutations. After intrauterine challenge with wild-type virus, mutated viruses, and cytopathogenic BVDV, all viruses could be detected in fetal tissue after 5, 7, and 14 days. Type 1 interferon (IFN) could be detected in fetal serum after challenge, except with wild-type noncytopathogenic BVDV. On days 7 and 14 after challenge, the largest quantities of IFN in fetal serum were induced by the N^{pro} and RNase-negative double mutant virus. The longer duration of fetal infection with the double mutant resulted in abortion. Therefore, for the first time, we have demonstrated the essential role of both N^{pro} and E^{rns} RNase in blocking interferon induction and establishing persistent infection by a pestivirus in the natural host.

Bovine viral diarrhea virus (BVDV) is a member of the genus *Pestivirus* within the family *Flaviviridae*, which also contains the genera *Flavivirus* and *Hepacivirus* (19). Other members of the genus are the important animal pathogens *Classical swine fever virus* and *Border disease virus* of sheep. Pestiviruses are single-stranded, positive-sense RNA viruses with genomes of ~12.3 kb that contain one long open reading frame (ORF) coding for a polyprotein of about 4,000 amino acids, which is co- and posttranslationally processed into at least 12 mature proteins (30, 32). The proteins C, E^{rns}, E1, and E2 are structural components of the virion (55, 62). Both E^{rns} and E2 induce neutralizing antibodies in infected animals (60, 61) and elicit protective immunity (25, 27, 48, 58).

Cytopathogenic (cp) and noncytopathogenic (ncp) biotypes of all pestivirus species can be differentiated during replication in tissue culture cells (28, 32, 39). According to recent publications, the cp phenotype is characterized by a loss of control of genome replication and a reduced ability of the infected cell to prevent a type I interferon (IFN) response to double-stranded RNA (dsRNA) (2, 29, 30, 52).

With regard to genome organization, strategy of gene expression, biochemical properties, and functions of viral proteins, pestiviruses exhibit striking similarity to human hepatitis C virus (32). The most obvious difference between the viruses at the genome level is the presence of two additional protein coding regions in the pestivirus RNA. These sequences code for the nonstructural protein N^{pro} and the viral envelope protein E^{rns}. N^{pro} represents the first protein encoded by the long pestivirus ORF. It exhibits protease activity and is not essential for virus replication in tissue culture cells (16, 47, 56). N^{pro} has been reported to interfere with the host cellular IFN response to different stimuli, for example, infection with different viruses or treatment with dsRNA (16, 45, 46). Deletion of the complete N^{pro} coding sequence from the genome of classical swine fever virus resulted in reduced growth rates and attenuation in the natural host (33, 56).

The E^{rns} protein represents an essential component of the pestivirus particle. Deletion of the E^{rns} coding region from the viral genome resulted in replicons capable of autonomous RNA replication but unable to produce infectious virus particles (63; G. Meyers, unpublished results). In addition to its function as a structural protein, E^{rns} has the unique feature of containing an intrinsic RNase (18, 22, 50, 64), whose active site exhibits sequence homology with RNase Rh, a member of the T_2/S RNase superfamily (20, 22, 50). The protein forms a disulfide-linked homodimer of about 90 kDa, nearly half of which is due to glycosylation (27, 49). E^{rns} lacks a typical transmembrane region and accomplishes its association with the viral envelope by an as yet unknown mechanism dependent on its utmost C-terminal region (14, 23, 49). The protein is not only part of the viral envelope but is also secreted in consid-

* Corresponding author. Mailing address: Institut für Immunologie, Friedrich-Loeffler-Institut, Paul-Ehrlich-Str. 28, D-72076 Tübingen, Germany. Phone: 49 7071-9670. Fax: 49 7071-967303. E-mail: gregor.meyers@fli.bund.de.

† Present address: BioScreen European Veterinary Disease Management Center GmbH, Mendelstr. 11, Building L1, D-48149 Münster, Germany.

‡ Present address: Boehringer Ingelheim Vetmedica GmbH, D-55216 Ingelheim am Rhein, Germany.

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TABLE 1. Designations and properties of viruses recovered from full-length cDNA constructs

Mutant virus	cDNA construct	Parental virus	RNase[a]	N^{pro}[a]	Residual N^{pro} sequence[b]
XIKE-A	pK40A	BVDV-2/NY	+	+	Full length
XIKE-A-NdN	pK87C	BVDV-2/NY	+	−	MELF
XIKE-B	pK40B	BVDV-2/NY	−	+	Full length
XIKE-B-NdN	pK88C	BVDV-2/NY	−	−	MELF
KE9-A	pKE9	BVDV-1/KE9	+	+	Full length
KE9-A-NdN	pKE9/N−	BVDV-1/KE9	+	−	MELI
KE9-B	pKE9/R−	BVDV-1/KE9	−	+	Full length
KE9-B-NdN	pKE9/N−/R−	BVDV-1/KE9	−	−	MELI

[a] −, deletion of codon 349 (RNase) or deletion of N^{pro}.
[b] The residual N^{pro} amino acid sequence is indicated using the one-letter code. Full length, no deletion.

erable amounts into the extracellular space (14, 49). A role of E^{rns} in virulence and pathogenicity is strongly suggested by the fact that recombinant pestiviruses in which the RNase activity of E^{rns} is knocked out are clinically attenuated (35, 37). A role of E^{rns} and its RNase in the interaction of the virus and the immune system of the host or the host cell has been proposed (26, 35, 37). Recently, E^{rns} was shown to interfere with the type I IFN response of cells to dsRNA, and this activity was dependent on the RNase activity and a recently described capacity of the protein to bind dsRNA (26).

The definitive functions of the N^{pro} protein and the E^{rns} RNase activity have so far not been clarified. Deletion of N^{pro} or abrogation of the RNase activity results in viable viruses that are able to replicate in tissue culture cells and in natural hosts (16, 24, 33, 35, 37, 56). Accordingly, the necessity for conservation of these two features during evolution is not well defined.

In the field, pestiviruses establish persistent infections in their natural hosts (41, 54). This is best understood for BVDV, where a prerequisite for virus persistence is the infection of pregnant cows with an ncpBVDV during the first ca. 3 months of gestation (4, 7, 39). In contrast to ncpBVDV, cp viruses are not able to establish persistent infection (6, 8), and this difference has been hypothesized to exist because cp viruses induce a solid type I IFN response in the fetus, whereas ncp viruses do not (9).

Both N^{pro} and the E^{rns} RNase have been reported to interfere with the type I IFN response in cells infected with different viruses or treated with dsRNA (16, 26, 31, 45). It was therefore tempting to analyze whether one of these proteins is involved in the establishment of persistent infections. To this end, we generated different BVDV mutants, used these viruses for infection of pregnant heifers, and analyzed whether the viruses induced an IFN response and established infection in the fetuses.

MATERIALS AND METHODS

Cells and viruses. MDBK cells were obtained from the American Type Culture Collection (Rockville, MD). Individual cell clones were prepared from these cells by end-point dilution, and clone B2 was selected for further work because of its superior properties in transfection experiments. MDBK-B2 cells were shown to mount a type I IFN response after, e.g., infection with cytopathogenic BVDV (not shown). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (tested for the absence of pestivirus and antibodies against pestiviruses) and nonessential amino acids.

BVDV type 1 (BVDV-1) strain KE9 was isolated during a routine screening of German BVDV field strains. It was selected for further work because it is efficiently transmitted to fetuses in pregnant animals. Viruses XIKE-A and XIKE-B, derived from BVDV New York'93 (field isolate VLS#399), were described before (35). Cytopathogenic BVDV Pec515 has been described previously (7).

Infection of cells and immunofluorescence assay. Since pestiviruses tend to be associated with their host cells, lysates of infected cells were used for infection. Lysates were prepared by freezing and thawing cells 3 to 5 days after infection and were stored at −70°C. Unless indicated otherwise in the text, a multiplicity of infection (MOI) of 0.1 was used for infection of culture cells.

BVDV infection of MDBK-B2 cells was monitored by indirect immunofluorescence analysis with monoclonal antibody 8.12.7, directed against pestivirus NS3 (11). The cultures were washed twice with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde-2% glutaraldehyde in PBS for 20 min at 4°C, and then washed again with PBS. Permeabilization of the cells was achieved by the addition of 0.1% saponin in PBS for 5 min at 4°C. After three washes with PBS, bound antibodies were detected with a fluorescein isothiocyanate-conjugated goat anti-mouse serum (Dianova, Hamburg, Germany).

PCR and RT-PCR. PCR was carried out either with *Pfu* polymerase (Promega, Mannheim, Germany) or with *Taq* polymerase (Appligene, Heidelberg, Germany), following the manufacturer's recommendations and using ca. 50 to 100 ng of DNA template and 25 pmol of each primer. Reverse transcription-PCR (RT-PCR) was done with the One Step RT-PCR system (QIAGEN, Hilden, Germany), using 2 μg of total RNA as a template and following the manufacturer's instructions. The amplified PCR products were purified by preparative agarose gel electrophoresis and elution with a Nucleospin II kit (Macherey-Nagel, Düren, Germany) as recommended by the manufacturer.

Construction of BVDV-2 N^{pro} deletion mutants. In PCRs with primers CM172, CM173, CM174, and CM189 (each together with CM175), cDNA fragments containing N^{pro} deletions with the 5′-terminal one, three, four, or six codons of the N^{pro} gene preserved were generated and inserted into pKANE22A, a cDNA clone corresponding to the 5′-terminal part of the BVDV New York'93 genome. After being sequenced, the mutated cDNA fragments were inserted into the full-length construct pKANE40A via EagI and PmlI digestion, resulting in the constructs pK87A, -B, -C, and -D, respectively. Similarly, the full-length construct pK88C, which carries the same N^{pro} deletion as pK87C together with the RNase-inactivating mutation H349Δ, was established by insertion of the respective N^{pro} deleted fragment via the same enzymes into pKANE40B (35). Basic features of the cDNA constructs and the resulting viruses are presented in Table 1. Further details of the cloning procedures are available upon request.

cDNA cloning and nucleotide sequencing. Five micrograms of RNA from MDBK-B2 cells infected with BVDV KE9 (passage no. 8) was used for cDNA synthesis as described before (38). For second-strand synthesis, a Time Saver cDNA synthesis kit (GE Healthcare Europe, Freiburg, Germany) was used as recommended by the supplier. After ligation of EcoRI/NotI adaptors (Time Saver kit), the cDNA was used to select fragments with sizes above 3 kb by preparative gel electrophoresis as described before (36). Ligation with phage ZAPII DNA (EcoRI and calf intestine alkaline phosphatase treated; Stratagene), packaging of phage DNA into particles (Gigapack Gold; Stratagene), and plating of phage were done as described by the supplier. Screening of the library was done as described before, using the complete insert of the full-length clone pA/BVDV (38) as a probe. Positive phage clones were isolated, and plasmids were recovered by in vivo excision as recommended by Stratagene.

RT-PCR was used to obtain cDNA fragments downstream of nucleotide position 10,000 in the viral genome. RT-PCRs were conducted with the following primers: PCR 1, Ol-KE9/4 and Ol-KE9/4R; PCR 2, Ol-KE9/4 and Ol-KE9/5R;

PCR 3, Ol-KE9/5 and Ol-KE9/6R; PCR 4, Ol-KE9/5 and Ol-KE9/7R; PCR 6, Ol-KE9/8 and Ol-KE9/11R; PCR 7, Ol-KE9/8 and Ol-KE9/12R; and PCR 12, Ol-KE9/10 and Ol-KE9/11R (primer sequences are available upon request).

Determination of the 3′-terminal sequence via ligation of a DNA oligonucleotide to the RNA was done as described before (35). RT-PCR was conducted with primers KE9/3′P and KE9/3′R and 25% of the product of the ligation reaction. Thereafter, a nested PCR was conducted with primers KE9/3′Pn and KE9/3′PrN. Sequencing of the PCR product revealed a 3′-terminal sequence ending with four C residues. This result fits well with published data showing that the pestivirus genomic 3′ end is somewhat variable, with three to five terminal C residues (5, 12, 40, 42, 44).

Nucleotide sequencing was done with a primer walking strategy (primer sequences are available upon request), using a BigDye Terminator cycle sequencing kit (Perkin-Elmer, Applied Biosystems, Weiterstadt, Germany) and an ABI Prism 377 DNA sequencer (Perkin-Elmer, Applied Biosystems). Sequence analysis and alignments were done with Genetics Computer Group software (13).

Construction of the BVDV-1 KE9 full-length cDNA clone and establishment of KE9 mutants. Starting with the cloned cDNA and the PCR fragments, the full-length cDNA clone pKE9 was established according to standard procedures. The 5′- and 3′-terminal sequences were established by PCRs. For the 5′ end, a first PCR was conducted with primers Ol-KE9/5′voll and Ol-KE9/1R, using the cDNA clone pKE9-11/8 as a template. The 5′-terminal sequence contained in Ol-KE9/5′voll represents the most closely related sequence identified in sequence comparison studies with published BVDV sequences. Subsequently, a T7 RNA polymerase promoter was introduced via further PCR procedures. The 3′ end was established with a PCR using the product of the successful 3′-end RT-PCR described above as a template, together with primers Ol-KE9/3Pn and Ol-KE9/3′Srf. The resulting fragment contains an SrfI restriction site for linearization of the full-length clone prior to runoff transcription. Cloning was done via AatII and an XbaI site that was also introduced by the Ol-KE9/3′Srf primer.

An RNase-negative mutant of the full-length clone with a deletion of codon 349 was established via a PCR-based approach with primers Ol-KE9/11 and Ol-KE9d349R. Similarly, an N^{pro} deletion construct corresponding to pK87C and a double mutant equivalent to pK88C with a deletion in the N^{pro} coding region and the RNase-inactivating mutation described above were generated using PCR and standard cloning procedures. The basic characteristics of the constructs and the names of the recovered viruses are presented in Table 1. All primer sequences and additional details of the cloning procedures are available upon request.

Maternal infection experiments with pregnant heifers. Animal experiments with infection of pregnant animals were conducted according to our protocols in the Bár Animal Experiment Facility, Mohács, Hungary, by staff of the Veterinary Medical Research Institute, Budapest, Hungary, in a facility where the animals were kept strictly isolated. Briefly, pregnant heifers from a herd selected for this study because of the lack of a history of abortive diseases and who tested free of BVDV and BVDV antibodies were inoculated between days 60 and 90 of gestation, either intranasally or intramuscularly, with the indicated amounts of the different viruses. During the first 14 days postinfection (p.i.), the animals were examined daily for signs of disease (especially raised body temperature, respiratory abnormalities [abnormal respiratory rate, nasal or ocular discharge, conjunctivitis, sneezing, or coughing], reduced appetite, and diarrhea). Blood was taken for leukocyte counts and buffy coat preparation. Buffy coats were used for detection of viremia as described before, with the exception that one blind passage was carried out before detection of viral antigen (35). On the day of challenge, the day of study termination, and days 14, 28, and 42 p.i., sera were prepared and tested for the presence of BVDV neutralizing antibodies (35).

The animals were euthanized at 2 months p.i. according to currently practiced routine procedures. Fetuses and fetal material were extracted immediately. Fetuses were necropsied by an experienced veterinary pathologist, findings were recorded, and the following panel of samples was collected: exudate from the peritoneal cavity or thorax (if present), spleen, a piece of small intestine (corresponding to the area of the Peyer's patches), distal ileocecum, mesenteric lymph nodes (if enlarged), kidney, thymus, bone marrow from the sternum, cerebellum, and placenta (if available).

Tissue suspensions were made in a mortar, using sterile sea sand and 2 ml of ice-cold PBS-A (PBS without calcium or magnesium). Mortars were rinsed with 1 ml ice-cold PBS-A. The suspensions were centrifuged for 10 min at 2,000 × *g* at 4°C. The supernatant was cleared by passage through a disposable 0.45-μm filter holder, followed by passage through a 0.2-μm filter. Two hundred microliters of the supernatant was used for virus isolation, which was carried out in duplicate on a monolayer of MDBK-B2 cells in a 24-well tissue culture plate. After 1 hour of incubation with the inoculum, supernatants were replaced with fresh culture medium. Tissue cultures were checked daily for cytopathic effects or microbial contamination. After an incubation time of 5 days, plates were frozen and thawed twice, and 100 μl of tissue culture suspension was passaged on MDBK-B2 cells. After an additional 5 days of incubation, the virus was detected by indirect immunofluorescence staining.

In utero infection. Eleven BVDV antibody-negative cows were presented for in utero infection at approximately 60 days of pregnancy (range, 58 to 70 days). A laparotomy was performed on each of the cows as described previously (9), and 10 ml of amniotic fluid was removed by aspiration, using an 18-gauge needle. Five milliliters of the appropriate challenge material was injected directly into the amniotic fluid. Three animals were injected with 5×10^6 50% tissue culture infective doses ($TCID_{50}$) of XIKE-A-NdN (N^{pro} deleted), three were injected with 5×10^6 $TCID_{50}$ of XIKE-B (E^{rns} RNase negative), three were injected with 5×10^6 $TCID_{50}$ of XIKE-B-NdN (N^{pro} deleted/RNase negative), one was injected with XIKE-A (wild-type ncpBVDV), and one was injected with Pec515 (cpBVDV). One animal from each group of XIKE-A-NdN-, XIKE-B-, and XIKE-B-NdN-infected animals was killed on days 5, 7, and 14 postchallenge. The animals challenged with XIKE-A and cpBVDV were killed on day 7. Samples of amniotic fluid, fetal spleen, fetal liver, and fetal blood were harvested at postmortem examination and stored at −70°C. Maternal blood samples were collected daily pre- and postchallenge.

Type I IFN assay. Levels of biologically active type I IFN were assayed in triplicate in samples of serum and amniotic fluid by using an Mx promoter-chloramphenicol acetyltransferase reporter assay (15).

Western blotting. The levels of IFN-induced Mx protein in fetal liver samples were determined as an alternative indicator of IFN induction. Homogenates of fetal livers were prepared, and the protein concentration of each sample was determined (BCA protein assay kit; Pierce). An equivalent quantity of protein from each sample was suspended in 15 μl of electrophoresis sample buffer, resolved under reducing conditions, and transferred to an enhanced chemiluminescence nitrocellulose membrane, as described previously, for the preparation of nitrocellulose-bound antigen (10). After blocking of the membrane with 5% (wt/vol) semiskim milk in PBS containing 0.1% (vol/vol) NP-40, the membranes were probed and processed for ECL visualization of proteins according to the manufacturer's instructions (Amersham). The Mx protein was detected using a rabbit antiserum raised against human MxA (serum 49; a gift from P. Staeheli, Freiburg, Germany) at a dilution of 1:800 and horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (IgG; Sigma-Aldrich) at a dilution of 1:2,500.

Nucleotide sequence accession number. The BVDV KE9 sequence has been deposited in the GenEMBL data library under accession number EF101530.

RESULTS

Wild-type BVDV XIKE-A and RNase-negative mutant XIKE-B infect fetuses at high frequencies. BVDV-2 recombinants XIKE-A (wild type) and XIKE-B (RNase negative) were recovered from infectious cDNA clones derived from the highly pathogenic BVDV-2 strain New York'93. XIKE-A was shown to be highly pathogenic for calves, whereas the mutant XIKE-B induced only mild signs of disease, although it showed wild-type growth in tissue culture (35). To test whether these viruses cross the placenta and are able to establish persistent fetal infection, two groups of five pregnant heifers were inoculated intranasally with 10^5 $TCID_{50}$ of either XIKE-A (group 1) or XIKE-B (group 2). The animals in group 1 showed clear signs of disease, for example, fever, leukopenia, loss of appetite, respiratory symptoms, and diarrhea (data not shown). One animal was euthanized on day 12 p.i., and the fetus was recovered for further analysis. One animal aborted at 6 weeks p.i. The remaining animals were euthanized at 8 weeks p.i. Postmortem examination showed that one animal (no. 526) contained no fetus; only the remains of an autolyzed placenta were found, indicating either undetected abortion or reabsorption of the fetus. In the other two animals (no. 598 and 618), the fetuses were dead and showed general autolysis, indicating that they died ca. 2 to 3 weeks before the postmortem, respectively. Samples from different tissues of the extracted or aborted

TABLE 2. Detection of virus in fetuses from heifers infected with BVDV XIKE-A or XIKE-B

Animal no. and infecting virus[a]	Presence of virus[b]							
	Abdominal fluid	Small intestine	Spleen	Thymus	Kidney	Bone marrow (sternum)	Cerebellum	Placenta
XIKE-A								
615**§	NA	Neg	Neg	Neg	Neg	Neg	Neg	Neg
526*	NA	NA	NA	NA	NA	NA	NA	NA
626§	++	Neg	Neg	++	++	Neg	++	Neg
618**§	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
598**§	++	NA	++	++	++	++	Neg	Neg
XIKE-B								
565**	++	++	++	++	++	++	++	Neg
588	Neg	NA	Neg	Neg	Neg	Neg	NA	NA
608	NA	NA	++	++	++	++	++	Neg
469**§	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
619**§	NA	Neg	Neg	Neg	Neg	Neg	Neg	Neg

[a] *, no fetus found; **, abortion, and fetus was found; §, fetus showed autolysis.
[b] Each of the samples obtained from fetuses extracted at 2 months p.i. was tested twice. ++, virus was detected in both of the duplicate wells; Neg, no virus detected; NA, not available.

fetuses were recovered and analyzed for the presence of BVDV (Table 2). The fetuses from animals 626 and 598 were virus positive, whereas the samples obtained from animals 615 and 618 were negative. The latter result does not necessarily mean that the fetuses did not contain virus at the time of death, since the degree of autolysis was so high that inactivation of virus could be expected. Since death of the fetuses, abortion, and/or reabsorption was most likely due to BVDV infection, even when virus was not detected in the samples obtained from the animals, it can be concluded that in all five animals, transplacental infection of the fetuses occurred and caused extensive damage and/or persistent virus infection. Thus, XIKE-A represents a good starting point for testing mutations with regard to their influence on establishment of fetal infection.

As expected, only mild signs of disease were recorded for the animals in group 2, who were challenged with XIKE-B (RNase negative). No animals died, but again one animal (no. 469) aborted at 6 weeks p.i. The fetuses of the other four animals were recovered at 8 weeks p.i. and analyzed for virus. One fetus (no. 619) was dead with autolysis, indicating that it died 3 to 6 weeks before the postmortem. Two other fetuses showed abnormalities, for example, perirenal edema (no. 608) or ascites, degeneration of the liver, and general tissue edema (no. 565), whereas one appeared normal (no. 588). The virus was identified in the fetuses from animals 565 and 608 (Table 2). Once again, the high degree of autolysis in animals 619 and 469 was most likely responsible for the difficulty in detecting virus. Taken together, the results show that in four of five infected animals, the fetuses contained virus and/or died or were aborted, most likely as a consequence of BVDV infection. It therefore can be concluded that destruction of the RNase activity of E^{rns} does not prevent the establishment of fetal infection and damage to the fetus, even though abrogation of RNase activity leads to strong attenuation in the adult host animal (35, 37).

Deletion of most of the N^{pro} coding region from the genome of XIKE-A results in recovery of viable viruses. N^{pro} represents a nonessential protein that, because of its published role in interference with the innate immune system of the host cell, could have an important function in the establishment of persistent pestivirus infections (16, 45, 46, 56). To determine the effects of N^{pro} deletion on the outcomes of infections of pregnant animals, N^{pro} gene deletion mutants were established, starting with the full-length plasmid pK40A. It has been reported that the sequences downstream of the translation initiation codon of the pestivirus ORF are important for the translation efficiency and viability of the viruses (43, 53). Thus, deletion of the entire N^{pro} coding sequence could have deleterious effects on the viruses or at least reduce their replication efficiency. However, preservation of a considerable number of N^{pro} gene codons would result in capsid proteins with amino-terminal extensions that once again could have a major impact on virus viability. We therefore established several full-length constructs with different deletions and tested whether the corresponding virus mutants could be recovered. Viable viruses were obtained from the mutant plasmids containing three, four, and six 5′-terminal codons of the N^{pro} coding sequence. Initial tests showed that the virus with three residual N^{pro} codons grew to only low titers (not shown), and we therefore decided to continue the studies with the virus recovered from pK87C (XIKE-A-NdN) (Table 1), since it represented the virus with the smallest number of residual N^{pro} residues (four residues preserved) that showed acceptable growth characteristics, with only slight retardation compared to the wild-type virus (Fig. 1).

XIKE-A-NdN establishes persistent fetal infection despite N^{pro} gene deletion. Five pregnant animals were infected with the N^{pro} deletion mutant XIKE-A-NdN. For control purposes, three animals were infected with XIKE-B (RNase negative), since this virus showed efficient fetal infection and did not produce severe clinical signs in the dam. Infection was carried out with 10^5 $TCID_{50}$ of virus by intramuscular application. For both groups, only some animals showed mild clinical signs and leukopenia, with a reduction of white blood cell counts of >40% of the reference values determined before infection. Clinical signs seemed to be more frequent in those animals infected with XIKE-B, but because of the small number of animals used, this result was not statistically significant. Vire-

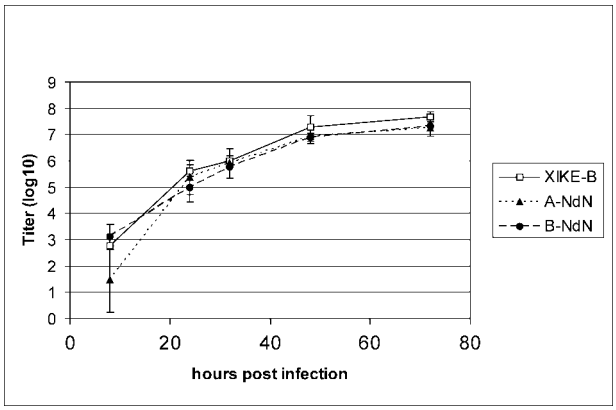

FIG. 1. Growth curves for the recombinant viruses XIKE-B, XIKE-A-NdN (A-NdN), and XIKE-B-NdN (B-NdN). XIKE-B represents an E^{rns} RNase-negative mutant of BVDV-2 New York'93 that was shown before to exhibit wild-type growth characteristics (35). MDBK-B2 cells were infected with the viruses at an MOI of 0.1 and harvested by freezing and thawing at the indicated time points. Titers were determined after infection of MDBK-B2 cells by immunofluorescence staining at 72 h p.i. The curves show the mean values for three independent experiments, with the standard deviation given for each time point.

mia was not detected in the infected animals. At 2 months postinfection, the animals were euthanized. For all three animals infected with XIKE-B, a fetus was present at postmortem, one of which (animal 1438) looked normal and did not contain infectious virus. The other two showed lesions, for example, serous fluid in the abdominal cavity accompanied with general edema of the tissues. All of the tissues tested, except for placentas, were virus positive for these fetuses (Table 3).

One of the animals infected with the N^{pro} deletion mutant XIKE-A-NdN did not contain a fetus, and no reason for the terminated pregnancy could be established (heifer no. 1637). One of the remaining four fetuses was normal, and virus was not detected in any of the tissues examined, whereas the other three fetuses showed abnormalities similar to those described above and were found to be virus positive in all tissues examined, except the placenta (Table 3).

The identity of the recovered viruses was verified by RT-PCR and nucleotide sequencing for at least one of the virus-positive samples from each of the fetuses. Thus, it can be concluded that similar to the case for RNase inactivation, the deletion of most of the N^{pro} coding sequence does not prevent the establishment of persistent infection in the fetus, although both mutations lead to considerable attenuation in adult animals.

BVDV-2 double mutant XIKE-B-NdN with inactivated E^{rns} RNase and deleted N^{pro} coding sequences does not result in fetal infection at 2 months p.i. Since the E^{rns} RNase and the N^{pro} protein have both been reported to be antagonists of the innate immune response in pestivirus-infected host cells (16, 26, 45, 46), the results of the experiments described above supported the hypothesis that a certain redundancy of the systems could compensate for the loss of either function and thereby allow establishment of fetal infection in our experimental system in the absence of either RNase activity or N^{pro}. To test this hypothesis, we combined both of the mutations in the full-length plasmid pK88C. A virus mutant was recovered from this construct that exhibited similar growth characteristics to the N^{pro} deletion mutant XIKE-A-NdN and thus showed some slight growth retardation compared to a virus containing the N^{pro} coding sequence in its genome (Fig. 1). This virus mutant was tested in two animal experiments. In the first experiment, five pregnant heifers were infected intranasally with 10^5 $TCID_{50}$ of the mutant. In the second study, nine pregnant animals were inoculated via the intramuscular route with 10^6 $TCID_{50}$. No clinical signs and no leukopenia were observed in the dams after challenge. Viremia was found in three animals from the second study (no. 4388, 4493, and 4559), but only on one day each and only in one of the duplicate samples (not shown). Thus, it could be hypothesized that the double mutant replicated only inefficiently in the heifers. However, all heifers seroconverted and developed significant neutralizing antibody titers (Table 4).

When the animals were euthanized at 2 months postinfection, all were found to contain fetuses with no signs of abnormal development. Analysis of fetal tissues revealed that all fetuses were free of virus. It therefore could be concluded that the double mutant was not able to establish persistent fetal infection upon maternal challenge.

TABLE 3. Detection of virus in fetuses from heifers infected with BVDV XIKE-B or XIKE-A-NdN

Animal no.	Presence of virus[a]							
	Abdominal fluid	Small intestine	Spleen	Thymus	Kidney	Bone marrow (sternum)	Cerebellum	Placenta
XIKE-B								
1438	NA	NA	Neg	Neg	Neg	Neg	Neg	Neg
1464	++	++	++	++	++	++	++	Neg
1585	++	++	++	++	++	++	++	Neg
XIKE-A-NdN								
1562	NA	NA	Neg	Neg	Neg	Neg	Neg	Neg
1583	++	Neg	++	++	++	+−	++	Neg
1596	++	++	++	+−	++	++	++	Neg
1621	++	++	++	++	++	++	++	Neg
1637[b]	NA	NA	NA	NA	NA	NA	NA	Neg

[a] Each of the samples obtained from fetuses extracted at 2 months p.i. was tested twice. ++, virus was detected in both of the duplicate wells; +−, only one of the duplicate wells was positive; Neg, no virus detected; NA, not available.
[b] No fetus was found.

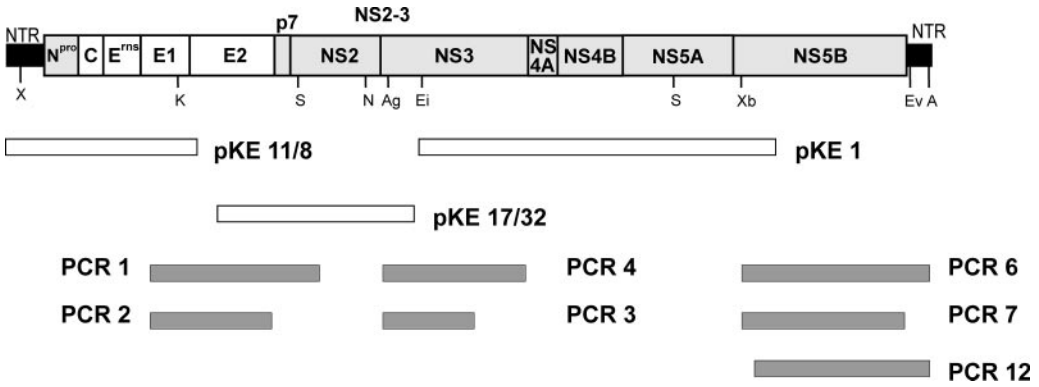

FIG. 2. Representation of the cDNA fragments used for construction of the infectious cDNA clone pKE9. The upper part sketches a BVDV genome and the encoded polyprotein (gray bars code for nonstructural proteins, white bars code for structural proteins, and black bars are nontranslated regions [NTR]). The middle and bottom parts show the cDNA clones (white) and the RT-PCR products (gray) used for engineering the infectious cDNA clone. Restriction sites used for cloning are as follows: X, XhoI; K, KpnI; S, SalI; N, NotI; Ag, AgeI; Ei, EcoRI; Xb, XbaI; Ev, EcoRV; and A, AatII. XhoI, KpnI, SalI, EcoRI, XbaI, and EcoRV cut more than once in the full-length clone.

Establishment of a BVDV-1 double mutant equivalent to XIKE-B-NdN. The above results could be specific for the mutant based on the BVDV-2 strain NewYork'93. We therefore wanted to establish an equivalent double mutant for another BVDV strain that did not produce overt clinical signs and was only distantly related to BVDV-2 New York'93. We therefore generated a new infectious full-length clone for the German BVDV-1 field isolate KE9 that exhibited only 70% sequence homology to the NewYork'93 RNA. We used the KE9 strain before as a challenge virus in experiments with pregnant heifers. In two independent experiments, a total of six of six fetuses extracted from heifers infected with KE9 were persistently infected (not shown).

A full-length cDNA clone was established with selected cDNA fragments (Fig. 2). The 5′- and 3′-terminal sequences were generated by PCRs which also served for the introduction of a T7 RNA polymerase promoter at the 5′ end and a unique SrfI site at the 3′ end. Infectious BVDV was recovered from the resulting full-length construct, pKE9, which showed growth characteristics very similar to those of the parental virus (Fig. 3).

In the second step, a BVDV KE9 genome with a deletion of most of the N^{pro} coding sequence was established. We decided to generate a mutant plasmid with a deletion preserving only the four 5′-terminal codons of the N^{pro} gene. This configuration is equivalent to the one in constructs pK87C and pK88C that allowed the recovery of the viruses XIKE-A-NdN and XIKE-B-NdN. Because of sequence variation, the mutant BVDV KE9 polyprotein derived from the resulting plasmid, pKE9/N−, starts with MELI, whereas the pK87C/pK88C-encoded proteins start with the residues MELF (Table 1). The resulting virus was named BVDV KE9-A-NdN. In addition, a construct with an RNase-inactivating mutation (pKE9/R−, with a deletion of codon 349) and a double mutant equivalent to the pK88C construct (pKE9/N−/R−, with deletion of histidine codon 349 of the ORF and the N^{pro} coding sequence) were established (Table 1).

TABLE 4. Titers of neutralizing antibodies in heifers infected with XIKE-B-NdN

Animal no.[a]	BVDV type 2 reciprocal titer (NY93/C)[b]			
	14 dpi	28 dpi	42 dpi	Termination of expt
921	<2	161	323	256
1013	<2	5	323	256
1015	5	161	323	323
1055	6	40	323	406
1075	<2	128	203	406
4387*	8	51	161	101
4388*	64	32	64	128
4467*	51	256	405	256
4491*	20	32	101	80
4493*	25	322	256	128
4499*	80	161	405	256
4518*	3	64	256	161
4543*	80	128	256	202
4559*	3	128	512	1,024

[a] *, animals from the second independent study.
[b] Results are expressed as the reciprocals of the serum BVDV-specific neutralizing antibody titers against ca. 10^2 $TCID_{50}$ of the heterologous BVDV-2 isolate KE13 or (*) BVDV-2 New York'93. All animals were seronegative at 0 days postinfection. dpi, days postinfection.

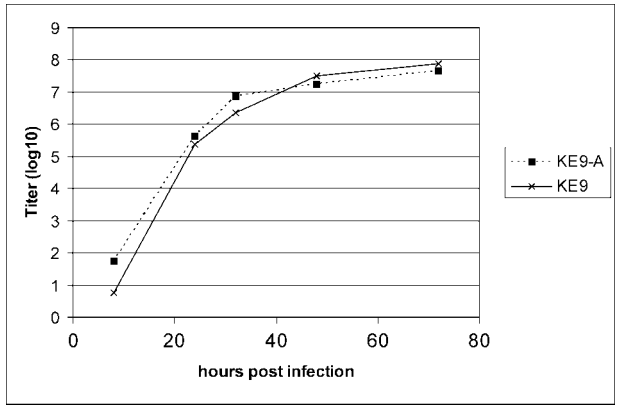

FIG. 3. Growth curves for wild type BVDV-1 KE9 and the recombinant virus KE9-A. Infection was done at an MOI of 1. Equivalent results were obtained with a total of three independent viruses recovered from plasmids isolated from three different bacterial clones. See also the legend to Fig. 1.

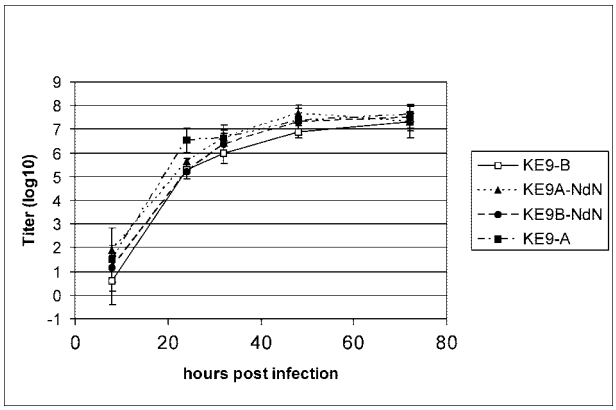

FIG. 4. Growth curves for the recombinant viruses KE9-B, KE9-A-NdN, and KE9-B-NdN. The original wild-type virus KE9-A recovered from the infectious clone served as a control. KE9-B represents an E^{rns} RNase-negative mutant of KE9-A, and the "NdN" versions contain the N^{pro} deletion in context with either wild-type E^{rns} (KE9-A-NdN) or RNase-negative E^{rns} (KE9-B-NdN). Infections were done at an MOI of 1. See also the legend to Fig. 1.

The mutant viruses were recovered from the cDNA constructs after in vitro transcription and RNA transfection. The genomes of the recovered viruses contained six deviations from the expected sequence resulting from changes in the cloned sequences or variation in cDNA fragments (CA2560/2561TC, A9054T, A9728G, C10192T [silent], and T10645C [silent]). The first of these mutations leads to a Gln-to-Ser change in a variable region of the E2 protein. The respective position is occupied by Val, Asn, and Glu in other pestivirus sequences. The second mutation results in an Asn-to-Ile change in the NS5A protein at a position where all the analyzed pestivirus sequences display Ile. The third mutation leads to an Ile-to-Val exchange at a position in NS5A where Leu is also found in other pestivirus proteins. Since the recovered virus KE9-A showed growth characteristics very similar to those of the parental virus (Fig. 3), a significant impact of these mutations on the viral phenotype is highly unlikely. All of the recovered viruses, including those with deletions in the N^{pro} coding region, showed similar growth rates (Fig. 4).

BVDV-1 KE9-B-NdN does not establish persistent fetal infection. Ten pregnant heifers were infected with 10^6 $TCID_{50}$ BVDV KE9-B-NdN via the intramuscular route. The infection did not result in signs of disease, fever, or significant leukopenia. Analysis of buffy coat samples did not result in detection of virus, but once again, the development of neutralizing antibody responses in all 10 heifers showed that the infections were productive (Table 5). At study termination at 2 months p.i., normal fetuses were found in all 10 heifers. Virus could not be detected in any of the analyzed fetal tissues. It therefore can be concluded that infection of pregnant heifers with double mutants lacking most of the N^{pro} coding sequence and expressing an RNase-negative E^{rns} protein does not lead to the establishment of persistent fetal infection, regardless of whether the infection is performed intranasally or intramuscularly.

Infection of fetuses by intrauterine challenge. The results of the experiments described above can be explained by the fact that the virus mutants do not get into the fetus because of a lack of systemic spread or that the mutations prevent the establishment or maintenance of persistent infection in the fetus. To discriminate between these two possibilities, further studies were conducted, in which viruses were injected directly into the amniotic fluid of pregnant animals. Earlier experiments with cp- and ncpBVDV indicated that the ability of the viruses to prevent a type I IFN response to viral infection represents a prerequisite for persistent infection (9). We therefore monitored IFN production in samples harvested at postmortem in addition to detection of virus infection in the fetuses. As a control for the IFN analysis, we infected the fetus in another animal with the same amount of a cpBVDV that was shown before to induce IFN production and to not establish persistent infection. The fetus in another animal was infected with the same dose of wild-type ncp virus XIKE-A, which was shown to infect fetuses at a high frequency. These two animals were euthanized on day 7 p.i., a time point that was identified in earlier experiments as optimal for IFN detection. For each of the mutants XIKE-B, XIKE-A-NdN, and XIKE-B-NdN, three animals were infected and were euthanized on days 5, 7, and 14 p.i.

At study termination, samples of fetal tissues and body fluids were removed. Type I IFN was measured in fetal serum and maternal serum samples. Fetal liver samples were collected to detect the presence of the Mx protein by Western blot analysis. A second set of fetal samples, including buffy coats, amnions, and allantoic fluid, and tissue material from livers, spleens, and kidneys were tested for the presence of virus by cocultivation of sterile filtered fluids or tissue extracts with MDBK-B2 cells in duplicate. After one passage, virus infection was demonstrated by indirect immunofluorescence. Negative results were verified by repetition of the analysis, starting with a second aliquot of the original material. For all fetuses expect one, virus could be detected in several samples. Only for animal no. 6, which had been infected with the N^{pro}-negative mutant XIKE-A-NdN, was just one virus-positive sample found (Table 6). The positive material was amniotic fluid, but only one well of two independent duplicate tests contained virus. This result might be due to technical problems or a failure to establish infection in this animal, because the samples obtained from the other animals infected with the same virus were positive. Thus, ani-

TABLE 5. Titers of neutralizing antibodies in heifers infected with BVDV KE9-B-NdN

Animal no.	BVDV type 1 reciprocal titer (KE9)[a]			
	14 dpi	28 dpi	42 dpi	Termination of expt
4384	13	80	256	202
4412	4	64	64	202
4454	40	202	512	405
4465	5	202	256	405
4500	5	161	1,024	405
4546	25	80	161	202
4555	20	256	809	644
4564	13	64	405	512
4570	25	405	1,024	405
4572	3	128	202	161

[a] Results are expressed as the reciprocals of the serum BVDV-specific neutralizing antibody titers against ca. 10^2 $TCID_{50}$ of BVDV KE9. All animals were seronegative at 0 days postinfection. dpi, days postinfection.

TABLE 6. Detection of virus in fetuses infected in utero with BVDV variants

Virus	Animal no. (day of termination)	Presence of virus[a]					
		Buffy coat	Amniotic fluid	Allantoic fluid	Liver	Spleen	Kidney
cpBVDV	1 (7)	+/−	−/−	NA	+/+	−/+	NA
XIKE-B-NdN	2 (5)	−/−	−/−	NA	+/+	+/+	+/+
	3 (7)	+/+	−/−	NA	+/+	+/+	NA
	4 (14)	§/+	−/−	NA	+/+	+/+	NA
XIKE-A-NdN	5 (5)	+/+	+/+	+/+	+/+	+/+	NA
	6 (7)	−/−	(a) −/−	NA	(a) −/−	−/−	(a) −/−
			(b) +/−	NA	(b) −/−		(b) −/−
	7 (14)	+/−	+/+	NA	+/+	+/+	NA
XIKE-B	8 (5)	−/−	+/−	NA	+/+	NA	+/+
	9 (7)	−/−	+/+	NA	+/+	+/+	NA
	10 (14)	+/−	+/+	NA	+/+	−/−	NA
XIKE-A	11 (7)	+/+	+/+	NA	+/+	+/+	NA

[a] Each sample was tested twice. +/+, virus detected; −/−, no virus detected; +/−, only one of the duplicate wells was positive; NA, not available; §, bacterial contamination, with no second analysis possible; (a) and (b), results of two independent assays conducted with the tissue sample.

mal no. 6 has to be regarded with caution when conclusions are drawn.

Virus titrations were performed on samples that were positive by virus isolation. The sample extracts were serially diluted before inoculation of the cells into 2 wells of 24-well plates for each sample. There was variation in the virus titers between different tissues of each fetus (Fig. 5). Interestingly, the most reliable source of tissue for virus detection seemed to be the liver, which was positive in all cases and contained high titers of virus except for fetus no. 6. The spleen was also positive in most cases but contained approximately 10^2 to 10^3 fewer infectious virus particles per gram of tissue. Virus isolation from buffy coat samples was variable, and therefore titrations were not performed.

A more thorough evaluation of the virus titers in liver samples showed that the largest amounts of virus were found in the fetus infected with the ncp wild-type control, whereas the titer of the cp virus was approximately 10^3 $TCID_{50}$/g lower. This finding fits very well with the fact that the cp virus, in contrast to the ncp virus, is not able to establish persistent infection in the fetus, which is thought to be due to the activation of innate immune responses by the cp virus (9). For all of the mutant viruses, titers were between the values determined for cp and ncp viruses. On day 14 p.i., the N^{pro} deletion single mutant XIKE-A-NdN reached the highest titer among the three mutants (Fig. 5).

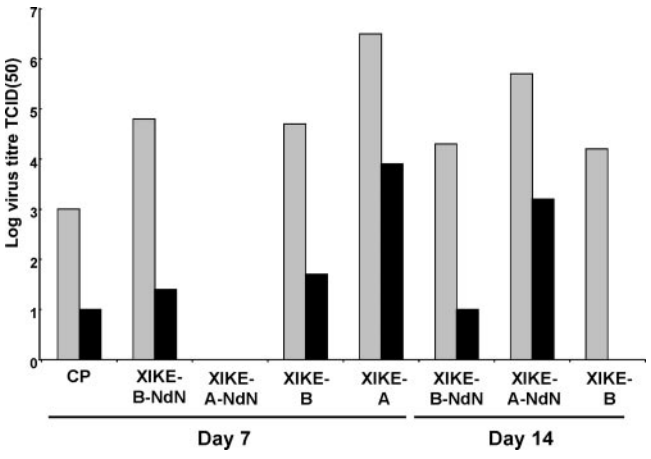

FIG. 5. BVDV viral titers ($TCID_{50}$) in 0.1-g fetal liver (gray bars) and spleen (black bars) samples at 7 and 14 days postchallenge. Tissue samples were obtained from fetuses challenged with the wild-type cytopathogenic BVDV (CP), wild-type noncytopathogenic BVDV XIKE-A, N^{pro}-negative noncytopathogenic BVDV XIKE-A-NdN, E^{rns} RNase-negative noncytopathogenic BVDV XIKE-B, or N^{pro}-negative/E^{rns} RNase-negative noncytopathogenic BVDV XIKE-B-NdN. Virus was detected in at least one sample from each animal (except the N^{pro}-negative virus at 7 days postchallenge for animal no. 6 [see the text for details]).

Analysis of biological IFN activity induced in the fetuses after infection with the cp and ncp controls reproduced the results obtained in earlier experiments, with no detection of IFN in the ncp XIKE-A-infected fetus and detection of IFN after cp virus infection. Interestingly, all three mutants induced IFN (except for the N^{pro}-negative virus in animal 6 7 days after challenge [see above]). The levels determined for the single mutants were comparable to those in cpBVDV-infected fetuses, whereas the double mutant induced much larger amounts of IFN (Fig. 6).

The expression of the IFN-inducible Mx protein in the fetal liver samples largely corroborated the fetal serum IFN results (Fig. 7). On day 5 p.i., similar quantities of IFN were detectable in each fetus, and equivalent expression of Mx was detected in the liver samples. Similarly, on day 14, the double mutant virus induced the largest quantity of IFN and the highest level of Mx expression. However, there were some inconsistencies in the correlation between IFN production and Mx expression in the fetuses extracted on day 7. The quantity of Mx expression in the liver of the cpBVDV-infected animal was greater than or equivalent to that of Mx expression in the livers of double mutant-infected fetuses, despite the fact that the double mutant virus stimulated approximately sixfold more IFN. Also, ncp virus clearly induces Mx expression, despite the absence of any IFN production. This is consistent with previous studies (9). In contrast, the Mx protein is not induced in vitro in response to ncpBVDV (51). Therefore, Mx induction in response to ncpBVDV in the early fetus may be via an IFN-independent pathway (reviewed in reference 51).

No Mx expression was detectable in samples from animal no.

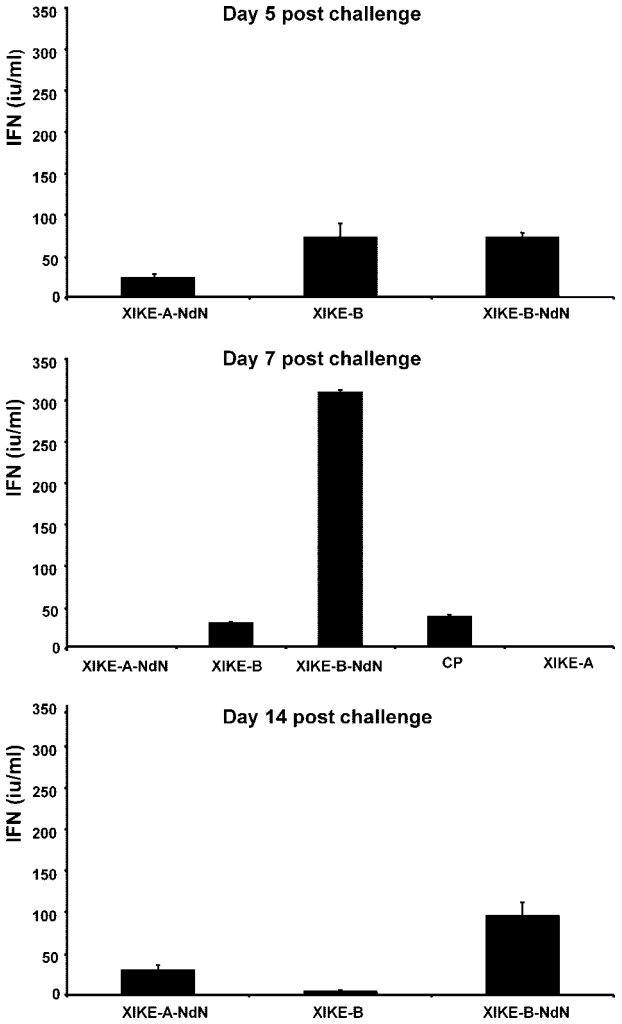

FIG. 6. Biological IFN activity (given in international units per ml [iu/ml]) was measured using MDBK cells transfected with an Mx promoter-chloramphenicol acetyltransferase reporter (15) in fetal serum samples at the indicated times after intrauterine BVDV challenge. Fetal serum samples were obtained from animals challenged with the wild-type cytopathogenic BVDV (CP), wild-type noncytopathogenic BVDV XIKE-A, N^{pro}-negative noncytopathogenic BVDV XIKE-A-NdN, E^{rns} RNase-negative noncytopathogenic BVDV XIKE-B, or N^{pro}-negative/E^{rns} RNase-negative noncytopathogenic BVDV XIKE-B-NdN. Each sample was analyzed in triplicate. Error bars are standard errors of the means.

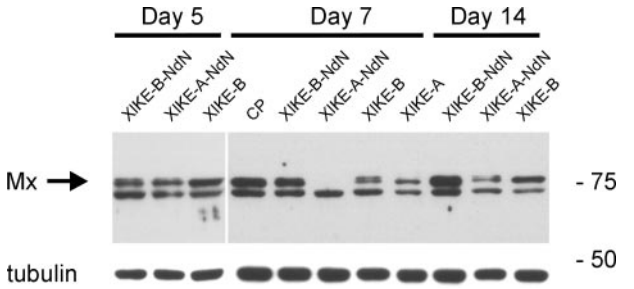

FIG. 7. Expression of IFN-induced protein Mx in fetal liver samples after intrauterine challenge with BVDV (5×10^6 $TCID_{50}$). The Mx protein was detected by Western blot analysis of homogenates of fetal livers from animals challenged with the wild-type cytopathogenic BVDV (CP), wild-type noncytopathogenic BVDV XIKE-A, N^{pro}-negative noncytopathogenic BVDV XIKE-A-NdN, E^{rns} RNase-negative noncytopathogenic BVDV XIKE-B, or N^{pro}-negative/E^{rns} RNase-negative noncytopathogenic BVDV XIKE-B-NdN. The Mx protein was detected as the higher-molecular-mass band of an approximately 75-kDa doublet. Equal quantities of total protein were loaded in each well; equal sample loading was confirmed by reprobing the blot with anti-tubulin antibody.

6, consistent with the inability to detect IFN in the serum of this fetus and a failure to detect significant viral growth.

The intrauterine infection experiment showed that all viruses, including the double mutant, infected and replicated in the fetus. Since the double mutant was not detected in the fetus after parental challenge, it was important to analyze whether this virus is eliminated after longer incubation following intrauterine challenge. Three pregnant heifers were infected with XIKE-B-NdN on day 74 of gestation according to the procedure described above. We intended to analyze fetuses for virus infection at about 2 months p.i. However, this experiment was interrupted because we observed abortion in all three heifers. The first abortion was observed in animal no. 2 on day 23 p.i. The other two heifers aborted on days 32 (animal no. 3) and 37 (animal no. 1) p.i. The aborted fetuses showed extensive autolysis. Attempts to detect virus via inoculation of tissue cultures with organ suspensions (using the same organs and procedure described above) or via nested RT-PCR failed.

DISCUSSION

Infection with ncpBVDV is common in the cattle populations of most countries worldwide (21). Crucial for the maintenance of the virus within herds is the ability of ncpBVDV to establish lifelong persistent infection following exposure to the virus in utero. Persistent infection occurs only if the fetus is exposed to virus prior to the onset of immune competence, at about 120 days of gestation; as a result, persistently infected animals do not exhibit detectable antibody or T-cell responses to the virus (10). Challenge with BVDV at later stages of pregnancy and postnatally, when the adaptive immune response has developed, usually results in transient infection.

Previous experimental studies have shown that in contrast to ncpBVDV, cpBVDV does not establish a persistent infection after fetal challenge during the first trimester of pregnancy (6). Further studies (9) showed that infection with cpBVDV Pec515 was associated with a strong type I IFN response, as indicated by the presence of biological activity in amniotic fluid. In contrast, there was no detectable IFN activity in the amniotic fluid of ncpBVDV-challenged animals, despite the finding that ncp virus replicated to higher levels than cp virus. It was proposed that the failure of cpBVDV to establish persistent infections was due to the induction of IFN production.

This difference in the capacity of ncp and cp viruses to induce an IFN response can also be demonstrated in tissue culture experiments and is apparently due to the ability of the ncp virus to block the dsRNA-induced IFN response (1, 2, 52). The fact that cp viruses cannot block the dsRNA-induced IFN response is most likely a consequence, at least in part, of their enhanced rate of RNA replication (3, 29, 30, 34, 59). Natural isolates of ncp viruses were shown to produce less RNA in

infected cells, and this seems to be a consequence of their absolute dependence on a cellular chaperone that is only present in limited amounts in the cell (29, 30). Both cp- and ncpBVDV need to replicate their RNAs, presumably via a double-stranded, so-called replicative form, and would therefore stimulate an antiviral response. However, for the ncp virus, the initial stimulating effect can be expected to be only temporary and weak because of the low level of RNA replication that occurs only during the first ca. 10 h p.i. (30). The predicted small quantities of dsRNA in ncp virus-infected cells should be an inefficient trigger for induction of an IFN response, which is obviously efficiently blocked by the viral antagonistic activities.

The nature of the mechanisms responsible for blocking the IFN response is still a matter of debate. Both the N^{pro} protein and the RNase activity of E^{rns} have been proposed to be involved (16, 26, 31, 45, 46). A recombinant E^{rns} protein has been shown to block dsRNA-induced IFN production in tissue culture cells. Also, the capacity of E^{rns} to block IFN production was abrogated by mutating the active site of the enzyme, thus inactivating the RNase activity (26). In addition, deletion of the majority of the N^{pro} coding sequence in different pestiviruses has been reported to prevent dsRNA-induced IFN production in tissue culture cells (16, 17, 31, 45). Similarly, expression of N^{pro} alone can block IFN induction by dsRNA (45).

Experiments with virus mutants in their natural hosts show that both alterations lead to virus attenuation (33, 35, 37). In the case of N^{pro} deletion, the connection between attenuation and N^{pro} function is difficult to prove since the complete deletion of the coding region often has a significant impact on virus growth (16, 33, 56; unpublished results). So far, the roles of the two activities during infection of the adult host are still obscure.

Since N^{pro} and the E^{rns} RNase have been shown to block IFN induction, we investigated whether these proteins have a role in the establishment of persistent infection in the fetus. In the first series of experiments, parental challenge of pregnant animals was conducted and showed that wild-type ncp virus and N^{pro}-negative and RNase-negative single mutants could be found in fetuses at 2 months p.i. In contrast, two different double mutant viruses could not be detected in fetuses after parental challenge. Interpreting the results of these studies is complicated because the virus mutants may have restricted growth in the dam, thus limiting the exposure of the fetus to viral challenge. The single mutant viruses may be less attenuated so that fetal infection may occur and persistent infection be established. In contrast, the double mutant virus is severely attenuated, probably due to the high levels of IFN induced at the site of inoculation. Hence, the pregnant uterus is possibly only exposed to very low titers of virus, and infection of the fetus does not readily occur. Interestingly, viremia was not routinely detected in the blood of the heifers after challenge with either the single or double mutants (data not shown). Thus, detectable maternal viremia is obviously not a prerequisite for fetal infection. With these results taken together, the failure to establish persistent infection after parental challenge may be due to critical attenuation of the virus, resulting in a failure to infect the fetus. Alternatively, the fetus may become infected but persistent infection does not occur due to the induction of IFN and elimination of the virus.

To discriminate between the two alternatives mentioned above, we conducted fetal challenge experiments and analyzed virus infection and the IFN response. These intrauterine challenges clearly showed that knocking out either N^{pro} or the RNase activity of E^{rns} results in IFN production in the early fetus. The double mutant virus, with N^{pro} and E^{rns} RNase activity deleted, induced IFN production in the early fetus, and strikingly, at day 7 postchallenge, there was greatly enhanced IFN production compared to that with either of the single mutant viruses or cpBVDV virus. Also, on day 14 p.i., IFN production was higher in the fetus infected with the double mutant than in single mutant-infected fetuses. Interestingly, in the present study, there was not a strict correlation between the capacity to establish persistent infection and the ability to prevent IFN induction. N^{pro}- and E^{rns}-mutated viruses and cpBVDV induced similar quantities of IFN in the early fetus; however, the mutated viruses were able to persist for at least 2 months in the fetus.

Importantly, all mutant viruses replicated in the fetus. In contrast to the single mutants, the double mutants were not detected in the fetus after maternal challenge. We therefore analyzed whether these viruses were eliminated after longer incubations following intrauterine challenge in three pregnant heifers. Abortion was observed in all three heifers at 3 to 5 weeks p.i. These results indicate that the absence of the double mutants from the fetuses after maternal infection was more likely due to the prevention of fetal infection by enhanced control of the virus in the adult animal than to its elimination from the fetus.

Abortion after intrauterine challenge with the double mutant may be a consequence of the very strong induction of the innate immune response in the fetus by this virus. Even though XIKE-A, the parental virus of the double mutant, is a highly pathogenic virus (35) and induces abortion (this study), we have shown that this virus does not induce IFN production in the early fetus. Therefore, wild-type XIKE-A has the capacity to induce abortion in the absence of IFN induction. In contrast, the double mutant is highly attenuated in the adult host (no leukopenia, no fever or clinical signs) but consistently induced abortion after intrauterine challenge. These results suggest either that the intrinsic capacity of XIKE-A to induce abortion is preserved or even enhanced in the double mutant virus or that abortion induced by the double mutant results from a different mechanism, most likely connected with the induction of high levels of IFN. Fetal pathology associated with the induction of high levels of IFN and other proinflammatory cytokines has been suggested for influenza virus infection of humans (57). Death of embryos in utero in connection with the induction of high levels of IFN-β was also reported for a mouse system (65).

The idea that the double mutant virus has an enhanced capacity to induce fetal death compared to wild-type virus due to strong IFN production is supported by the results of the single-mutant parental challenge studies. We have shown that the single mutants can persist for at least 2 months in fetuses without causing abortion or death, especially the N^{pro} single mutants, where no abortion was detected in any of the challenged animals. Thus, the double mutant is apparently more pathogenic for the fetus than the single mutants. These pathological observations are consistent with the double mutant

virus inducing higher concentrations of IFN than the single mutants.

Variation in the capacity of different virus isolates to cause abortion has been described previously. Abortion and fetal death were observed after intrauterine challenge with cpBVDV strain NADL at approximately 70 days of gestation (8). In contrast, a study by Brownlie et al. (6) showed that intrauterine challenge between 79 and 90 days of gestation with cpBVDV Pec515 did not result in abortion and that virus-negative calves were born. Our own unpublished observations agree with these findings, as cpBVDV Pec515 intrauterine challenge resulted in the birth of normal, virus-negative calves, indicating that differences in the outcomes of intrauterine challenge with cpBVDV might depend on the strain used for challenge. However, in the present study, we compare viruses that all derive from one parental strain and only differ by defined mutations. It therefore seems justified to conclude that the early abortion observed for the fetuses after intrauterine challenge with the double deletion mutant is a specific effect due to the loss of both N^{pro} and E^{rns} RNase functions.

The results of our experiments indicate that there is redundancy in the capacity of N^{pro} and E^{rns} to enable establishment of fetal infection after maternal challenge, since the absence of one of these functions alone was not sufficient to prevent the establishment of persistence. The analysis of the IFN responses of infected fetuses indicates that the two functions are additive, which fits into the concept that both functions contribute to the observed blockage of type I IFN induction and, in the end, control of innate immune reactions against the virus.

Establishing persistent infections is an essential component of the life cycle of BVDV in cattle populations. To enable a persistent infection, a fetus has to be infected in utero. We show here that viruses deficient in both the N^{pro} protein and the E^{rns} RNase activity are not able to achieve transplacental infection at a frequency detectable in our experimental setups, despite normal growth in tissue culture cells. Since both single mutants reached the fetus without showing significantly higher viremia, our results might reflect a loss of specific functions necessary for transplacental infection that most likely concern the inability of the double mutant to inhibit a strong local IFN induction.

As a further prerequisite for persistence, fetal challenge has to occur prior to the development of the adaptive immune response, resulting in tolerance to viral proteins. Furthermore, the virus has to prevent induction of the innate immune response, and published observations suggest that this is achieved by two mechanisms. Firstly, controlling viral RNA replication reduces the expression of dsRNA, a pathogen-associated molecular pattern, thereby preventing a burst of triggers for an immune response that would be difficult to control early in infection by viral proteins. However, the expression of pathogen-associated molecular patterns cannot be suppressed completely, so viral proteins are employed to block the induction of innate immune responses. In the current study, we demonstrate for the first time that both the E^{rns} and N^{pro} BVDV proteins are required to block IFN induction in the early fetus, hence enabling the establishment of persistent infection. Since N^{pro} was shown to block IFN induction within the infected cell, the secreted protein E^{rns} could be responsible for counteracting cross-priming of an IFN response by viral RNA. The complex model outlined here will not lead to very high incidences of persistent infection, and epidemiological data indicate that $<1\%$ of the host population is usually persistently infected. However, individual virus carriers can live for years, during which they continuously shed huge amounts of virus, so this strategy has to be regarded as highly successful.

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